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Survival, Biofilm Formation, and Growth Potential of Environmental and Enteric *Escherichia coli* Strains in Drinking Water Microcosms

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ABSTRACT

***Escherichia coli* is the most commonly used indicator for fecal contamination in drinking water distribution systems (WDS). The assumption is that *E. coli* bacteria are of enteric origin and cannot persist for long outside their host and therefore act as indicators of recent contamination events. This study investigates the fate of *E. coli* in drinking water, specifically addressing survival, biofilm formation under shear stress, and regrowth in a series of laboratory-controlled experiments. We show the extended persistence of three *E. coli* strains (two enteric isolates and one soil isolate) in sterile and nonsterile drinking water microcosms at 8 and 17°C, with T_{90} (time taken for a reduction in cell number of 1 $\log_{10}$ unit) values ranging from 17.4 ± 1.8 to 149 ± 67.7 days, using standard plate counts and a series of (reverse transcription-)quantitative PCR [(RT-)Q-PCR] assays targeting 16S rRNA, *tuf*, *uidA*, and *rodA* genes and transcripts. Furthermore, each strain was capable of attaching to a surface and replicating to form biofilm in the presence of nutrients under a range of shear stress values (0.6, 2.0, and 4.4 dynes [dyn] cm^{-2}; BioFlux system; Fluxion); however, cell numbers did not increase when drinking water flowed over the biofilm ($P > 0.05$ by *t* test). Finally, *E. coli* regrowth within drinking water microcosms containing polyethylene PE-100 pipe wall material was not observed in the biofilm or water phase using a combination of culturing and Q-PCR methods for *E. coli*. The results of this work highlight that when *E. coli* enters drinking water it has the potential to survive and attach to surfaces but that regrowth within drinking water or biofilm is unlikely.**

IMPORTANCE

The provision of clean, safe drinking water is fundamental to society. WDS deliver water to consumers via a vast network of pipes. *E. coli* is used as an indicator organism for recent contamination events based on the premise that it cannot survive for long outside its host. A key public health concern therefore arises around the fate of *E. coli* on entering a WDS; its survival, ability to form a biofilm, and potential for regrowth. In particular, if *E. coli* bacteria have the ability to incorporate and regrow within the pipe wall biofilm of a WDS, they could reinoculate the water at a later stage. This study sheds light on the fate of environmental and enteric strains of *E. coli* in drinking water showing extended survival, the potential for biofilm formation under shear stress, and importantly, that regrowth in the presence of an indigenous microbial community is unlikely.

Safe, clean drinking water is the foundation of society. However, even in developed countries, drinking water quality failures occur and have considerable public health impact. In particular, microbiological quality failures can be a significant threat to the supply of drinking water. The exact cause of these water quality malfunctions can be difficult to determine. In public water supplies, inefficient water treatment of the source could result in unwanted microorganisms entering water distribution systems (WDS). Within the WDS, there is potential for these organisms to regrow. For example, Nescerecka and colleagues (1) reported an order of magnitude increase in intact microbial cells in the effluent of a WDS, compared to the influent, which they attributed to regrowth supported by the presence of assimilable organic carbon (AOC). Alternatively, water main breaks provide an opportunity for microorganisms from the surrounding environment to enter the WDS at either the repair site or at leaky joints and other small gaps in the system when pressures are low (2).

While the ingress of microbes into the WDS is an immediate risk, it is the persistence of these microorganisms (including pathogens) within the system that poses a continued threat to water quality. This persistence may be due to the incorporation of these organisms into the pipe wall biofilm (3, 4, 5). Several studies have shown that in comparison to planktonic organisms in drinking water, fecal opportunistic pathogens can survive longer and have greater resistance to chlorine while in biofilms (6, 7). Pipe wall biofilm can therefore act as a reservoir for microorganisms, including *Escherichia coli* (8) and other pathogens (9). Sloughing of biofilm at a later stage could rerelease microbes into the bulk water, potentially causing water quality failures and a public health concern.

E. coli is the principal indicator for fecal contamination of drinking water, and by inference, its presence signifies the probability that fecal waterborne pathogens have entered the WDS. A substantial number of water quality failures are due to the detection of *E. coli* above regulation standards, i.e., surpassing the zero

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TABLE 1 Primer and TaqMan probe sets tested for targeting *E. coli* used in this study

Target	Primer or probe[a]	Sequence (5′-3′)	Annealing temp (°C)	Amplicon size (bp)	Reference
16S rRNA (total bacteria)	1369F	CGGTGAATACGTTCYCGG	60	123	72
	1492R	GGWTACCTTGTTACGACTT			
	Tm1389P	CTTGTACACACCGCCCGTA			
16S rRNA (*E. coli* specific)[b]	ECA75F	GGAAGAAGCTTGCTTCTTTGCTGAC	72	544	27
	ECR619R	AGCCCGGGGATTTCACATCTGACTTA			
tuf	TEcol553F	TGGGAAGCGAAAATCCTG	58	258	25
	TEcol754R	CAGTACAGGTAGACTTCTG			
	TEco573-T1-B1P	AACTGGCTGGCTTCCTGG			
uidA	uidAF	CAACGAACTGAACTGGCAG	60	130	24
	uidAR	CATTACGCTGCT			
	uidAP	CCCGCCGGGAATGC			
rodA	rodA984F	GCAAACCACCTTTGGT	60	120	26
	rodA984R	CTGTGGGTGTGGATTGACAT			
	rodA984P	AACCCCTACAACCGGCAGAATACC			

[a] The last letter of the primer or probe indicates whether it is a forward primer (F), reverse primer (R), or TaqMan probe (P).
[b] The assay used was a SYBR green Q-PCR assay.

tolerance policy per 100 ml water for *E. coli* in a WDS, implemented by state monitoring agencies (10, 11). Although *E. coli* is currently used as an indicator organism of a recent fecal contamination event, little is known of its fate once it enters drinking water and the WDS—how long can it survive, can it regrow in water, can it shelter or replicate within a pipe wall biofilm? Furthermore, the reliability of *E. coli* as a fecal indicator organism has been brought into question with reports of long-term survival of *E. coli* in soils, sediments, and water in both tropical and temperate regions (12, 13, 14, 15). Indeed, in many of these regions, *E. coli* is now recognized as part of the soil biota. The presence of soil-persistent *E. coli* (15) highlights a previously unconsidered source of contaminating *E. coli* from the surrounding soil in which the pipes are buried.

E. coli contamination of drinking water is traditionally detected by culture methods. These methods are simple to conduct and detect viable organisms. However, these methods are also slow, and colony counting incorporates only culturable bacteria that are capable of cell division at a rate that is sufficient to form colonies (16). In many instances, the number of viable organisms may be underrepresented by culturing due to the fact that uncultivable bacteria (17), sublethally damaged bacteria (18), and bacteria that are in a viable but nonculturable (VBNC) state (8, 19) will not be detected. In contrast, molecular techniques can be used to detect and monitor subtle temporal and spatial changes in *E. coli* dynamics within a mixed microbial community without the need to culture in a fast, sensitive, and accurate manner. Quantitative PCR (Q-PCR) is particularly suited for targeting and quantifying specific organisms within a mixed microbial community (20, 21). A major disadvantage of conventional nucleic acid-based techniques targeting DNA is that it does not differentiate between viable organisms and those that have been inactivated. Viability in the context of risk assessment for any pathogen or indicator organism is a critical aspect of detection, since nonviable organisms do not represent a health risk. RNA is indicative of viable organisms, as RNA is a labile molecule with a short half-life. The half-life of mRNA in *E. coli* has been shown to be less than 6.8 min (22, 23), indicating that mRNA should not persist for long in nonviable microorganisms and is therefore a potential molecular indicator of viable cells.

This study addresses the survival, biofilm formation under shear stress, and potential for regrowth of *E. coli* in drinking water and pipe wall biofilms. These issues are important to interpret microbiological monitoring data and safety of drinking water. Water quality failures due to the survival or regrowth of *E. coli* within a WDS bring into question the suitability of *E. coli* as an indicator organism of fecal contamination. To achieve this, we first evaluated a number of current gene targets (24, 25, 26, 27) and Q-PCR assays for *E. coli*. Furthermore, we expand their use by developing corresponding reverse transcription-quantitative PCR (RT-Q-PCR) assays to detect mRNA and explore their use as indicators of viable organisms. Subsequently, the fate of three *E. coli* strains (one soil strain and two commensal strains) after inoculation into chlorinated drinking water was investigated. Specifically, we determined the survival of *E. coli* at different temperatures in sterile and nonsterile drinking water via plate counts, Q-PCR, and RT-Q-PCR. We then asked whether the chosen *E. coli* strains have the ability to form biofilms under shear stress values similar to those that would be encountered within the WDS using the BioFlux 1000Z (Fluxion Biosciences, CA) system. Finally, we asked whether the *E. coli* isolates selected can regrow within drinking water microcosms with pipe wall material present (either free-living or in a biofilm on the pipe wall). To this end, we aimed to expand current knowledge on the survival, biofilm potential, and growth of *E. coli* in chlorinated drinking water to deliver a new understanding of this fecal indicator organism's response in the event of an ingress occurring in a WDS.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. To develop (RT-)Q-PCR assays to quantify *E. coli* in drinking water, a number of primer sets from the literature were selected and further evaluated for specificity by PCR and Q-PCR (Table 1). A specificity test panel of 34 bacterial strains (see Table S1 in the supplemental material) was con-

structed, including 11 *E. coli* strains isolated from soil, 2 fecal *E. coli* isolates, 3 *Shigella* strains, and other non-*E. coli* related species such as *Enterobacter*, *Citrobacter*, and *Staphylococcus aureus*. Each bacterial strain was inoculated separately from individual glycerol stocks into Luria-Bertani (LB) broth and incubated overnight (18 h) at 37°C. The bacterial culture that had grown overnight was streaked onto LB agar plates to ensure that individual colonies would form, and the plates were incubated overnight (18 h) at 37°C. Subsequently, colony PCR was performed by inoculating a single overnight colony into 1 ml of Tris-EDTA (TE) buffer (Sigma-Aldrich, Wicklow, Ireland) and heating to 95°C for 10 min. Two microliters of boiled colony lysate was used as the template to amplify the 16S rRNA gene using universal primers F63 (5′-CAGGCCT AACACATGCAAGTC-3′) (28) and R518 (5′-GTATTACCGCGGCTGC TGG-3′) (29) to ensure that the cell had lysed prior to testing the *E. coli*-specific primers (Table 1). PCR conditions were as follows: 2 μl of cell lysate was added to a 50-μl PCR mixture containing 1× PCR buffer (Sigma-Aldrich, Wicklow, Ireland), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.2 μM each primer, and 2.5 U of *Taq* polymerase (Sigma-Aldrich, Wicklow, Ireland). The reaction mixture was initially denatured at 94°C for 5 min, followed by 30 cycles of PCR, where 1 cycle consisted of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 7 min (Mastercycler gradient; Eppendorf, Hamburg, Germany). Subsequently, the *E. coli*-specific primers were tested against the specificity panel whereby the 16S rRNA gene-positive lysate was used as the template for Q-PCR amplification. PCR conditions were as described above, with the appropriate primer-specific annealing temperatures outlined in Table 1.

***E. coli* survival in drinking water. (i) Experimental setup.** *E. coli* strains Lys9 (soil isolate), J1 (fecal isolate), and SE11 (characterized enteric isolate) (30) were inoculated from glycerol stocks into 25 ml of LB broth and incubated, with shaking (200 rpm), at 37°C for 18 h. After incubation, the optical density (OD) was read on a Jenway 6300 spectrophotometer (Bibby Scientific Ltd., Staffordshire, United Kingdom). The starting numbers of *E. coli* per microcosm were normalized by inoculating 10^8 CFU, equivalent to an OD of 0.2, as determined from growth curves (data not shown) using the following formula:

$$\frac{\text{OD wanted (i.e., 0.2)} \times \text{microcosm volume (i.e., 15 ml)}}{\text{overnight OD}} \quad (1)$$

Prior to inoculation into each microcosm, *E. coli* cells were pelleted (Avanti J-20 XP; Beckman Coulter, CA, USA), and the supernatant was discarded. The pellet was resuspended in 10 ml phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl [pH 7.4]; Sigma-Aldrich, Wicklow, Ireland) and mixed thoroughly, before repelleting and repeating the PBS wash step twice to remove remaining LB broth from cells. Finally, cells were resuspended in 15 ml PBS, before aliquots (1-ml volumes) were added into each microcosm. The starting numbers of *E. coli* post-PBS washes were confirmed by enumeration on MacConkey agar. Washed and resuspended cells were serially diluted in PBS buffer from neat to 10^{-10}, in triplicate, and 10 μl of each triplicate dilution was plated on MacConkey agar before incubation overnight (18 h) at 37°C. Colonies from each 10-μl dilution were counted after incubation, multiplied by the appropriate dilution factor to determine the number of CFU per milliliter (CFU ml^{-1}), before the counts from the three replicates were averaged.

Six liters of drinking water was collected from the public supply at the National University of Ireland (NUI), Galway, Ireland. The chlorine concentration of the water sample was determined, in triplicate, using the chlorine Test 'N Tube kit (Hach, Dublin, Ireland) according to the manufacturer's guidelines. Half of this water was used to prepare the drinking water (DW) microcosms, while the other half was filtered through a 0.22-μm-pore-size filter, followed by autoclaving at 121°C for 15 min, to prepare filter-sterilized, autoclaved drinking water (FA-DW) microcosms. Microcosms were prepared to facilitate destructive sampling at 10 time points over a 70-day incubation by adding 15 ml of DW or FA-DW to 50-ml tubes. To each microcosm, 1 ml of the respective resuspended *E. coli* strain (10^8 CFU of Lys9, SE11, or J1) was individually inoculated into each 15 ml of DW or FA-DW. These microcosms were incubated at 8°C and 17°C, with shaking (200 rpm) for 70 days. These temperatures were selected as typical winter and summer temperatures in a temperate WDS (31). In total, 360 microcosms were set up per experimental treatment (i.e., 10 time points × 3 biological replicates × 2 temperatures × 2 water types × 3 *E. coli* strains). In addition, corresponding noninoculated controls were set up for each sampling time point. Microcosms were destructively sampled in triplicate on days 1, 2, 6, 13, 25, 33, 45, 52, 59, and 70. For each time point, survival of viable *E. coli* was determined by enumeration on MacConkey agar as described above. The T_{90}, defined as the time taken for a reduction in cell number of 1 $\log_{10}$ unit, was determined from CFU ml^{-1} at each time point for each *E. coli* strain in DW and FA-DW at both 8 and 17°C, using the equation $T_{90} = -t/\log_{10}(C_t/C_0)$, where C_0 is the CFU ml^{-1} at day 0, and C_t is the CFU ml^{-1} at day t (32). The average T_{90} was determined from three replicates at each time point.

On days 1, 25, 45, and 70, the remaining 14 ml of water from each microcosm incubated at 17°C was filtered onto a 0.22-μm filter and stored at −80°C for subsequent DNA and RNA extraction as described below.

(ii) DNA and RNA coextraction. DNA and RNA were coextracted from microcosms incubated at 17°C on days 1, 25, 45, and 70 using the AllPrep DNA/RNA minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions, with the following changes. Two hundred microliters of TE buffer and 20 μl of 10 mg ml^{-1} lysozyme were added directly to the filter. The lysis buffer was gently rotated over the filter for 1 h at 37°C. Filter lysate was then added to the spin column, and the manufacturer's instructions were followed. An additional DNase treatment was also included during the extraction process to remove contaminating DNA from the RNA fraction. For this, 10 μl of DNase I was mixed with 70 μl of RDD buffer (RNase-free DNase set; Qiagen, Hilden, Germany), added to each RNA preparation, and incubated at 20°C for 15 min. Finally, both DNA and RNA were added to the respective DNA or RNA AllPrep column. One hundred microliters of elution buffer (DNA) or 50 μl of RNase-free water (RNA) was added to the membrane in the column, incubated at room temperature for 5 min to ensure optimum elution of the DNA or RNA, and centrifuged at 8,000 rpm for 2 min. DNA and RNA were visualized on a 1% agarose gel to confirm successful extraction. To ensure the RNA fraction was free from contaminating DNA, RNA was diluted 1 in 10 in diethylpyrocarbonate (DEPC)-treated water, and 1 μl of either neat RNA or 10^{-1} RNA was used as the template in a 16S rRNA gene PCR using the F63 and R518 primers as outlined above.

(iii) Molecular quantification. 16S rRNA, *uidA*, *tuf*, and *rodA* genes and transcripts were quantified using the primer and probe sets outlined in Table 1. RNA was quantified via a two-step RT-Q-PCR. Prior to Q-PCR, cDNA was reverse transcribed from the RNA template using Superscript III (Thermo Fisher Scientific, Dublin, Ireland) as follows. Each reaction mixture contained 1 μl of 0.2 mM each dNTP, 1 μl of 50 μM random hexamer primers (Thermo Fisher Scientific, Dublin, Ireland), 7 μl nuclease-free water, and 5 μl sample RNA. Samples were denatured at 65°C for 5 min. Following denaturation, samples were placed on ice for 4 min. To each sample mixture, 4 μl of 5× first strand buffer, 1 μl of 0.1 M dithiothreitol (DTT), 1 μl RNase inhibitor (40 U μl^{-1}), and 1 μl Superscript III (200 U μl^{-1}) (Thermo Fisher Scientific, Dublin, Ireland) were added. Samples were incubated at 25°C for 5 min, 50°C for 50 min, and finally 72°C for 15 min. cDNA generated was used as the template for the subsequent Q-PCR assays. Q-PCRs, with either DNA or cDNA as the template, were conducted using the LightCycler 480 Probes Master mix (Roche, Penzberg, Germany) for *uidA*, *tuf*, and *rodA* TaqMan probe assays and the LightCycler 480 SYBR green 1 master mix (Roche, Penzberg, Germany) for 16S rRNA gene SYBR green-based assays. Briefly, for each 20-μl TaqMan Q-PCR assay, the following components were combined: 5 μl of nuclease-free water, 10 μl of 2× concentrated master mix, 1.8 μl of each 10 mM forward and reverse primer (Table 1), 0.4 μl of 10 mM TaqMan probe (Table 1), and 1 μl of sample DNA or cDNA. The following experimental run protocol was used: denaturation program of 95°C

for 10 min, amplification and quantification program of 95°C for 10 s and 60°C for 30 s, which was repeated for 40 cycles, and a hold step at 40°C. For each 20-μl 16S rRNA gene SYBR green reaction mixture, 8.8 μl of nuclease-free water, 10 μl of 2× concentrated hot start master mix, 0.1 μl of 10 mM forward and reverse primer, and 1 μl of sample DNA or cDNA were added. The experimental run protocol was as follows: denaturation program of 95°C for 5 min, amplification and quantification program (repeated for 40 cycles) of 95°C for 30 s, 72°C for 30 s, and 72°C for 5 s with a single fluorescence measurement. Once the run ended, a melting curve from 58 to 95°C, with a heating rate of 0.1°C per second, and a continuous fluorescence measurement, commenced. All (RT-)Q-PCR analyses were carried out using the automated analysis settings on the Roche LightCycler 480 instrument, and standard curve descriptors were reviewed and recorded for each reaction mixture.

(iv) (RT-)Q-PCR standard curves. DNA and RNA standard curves were constructed for the following genes: 16S rRNA, *uidA*, *tuf*, and *rodA*, using a serial dilution of a known quantity of appropriate DNA or cDNA, to extrapolate a value as described by Smith and colleagues (20). Briefly, the target gene was amplified from *E. coli* ATCC 23716 by PCR as outlined previously, and the PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned using the pGEM-T Easy vector systems (Promega, MyBio, Kilkenny, Ireland) according to the manufacturer's instructions. White colonies containing the vector and target amplicon insert were selected for colony PCR, inoculated into 100 μl of LB ampicillin (100 μg ml^{-1} ampicillin), grown for 2 h at 37°C, and subsequently, 1 μl was used as the template in a PCR mixture using the M13 forward vector primer (5′-TGTAAAACGACGGCCAGT-3′) and the target reverse primer (Table 1). This primer combination was selected to ensure an insert with an antisense orientation was selected. The colony PCR mixture and cycle were as described above. Amplicons of the correct size, incorporating a T7 promoter, were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

For the RNA standard curve, target RNA was generated from the PCR amplicons containing a T7 promoter, by *in vitro* transcription using the MEGAshortscript T7 transcription kit (Thermo Fisher Scientific, Dublin, Ireland) according to the manufacturer's instructions. Briefly, each *in vitro* transcription reaction (20 μl) contained 200 ng of PCR product, 7.5 mM each ribonucleotide, 1× T7 reaction buffer, and 1× T7 enzyme mix. The reaction mixture was incubated for 2 h at 37°C. DNA template was removed from the reaction mixture by treatment with 1 U of TURBO DNase (Ambion; Thermo Fisher Scientific, Dublin, Ireland) for 15 min at 37°C. The reaction was terminated by the addition of 115 μl of DEPC-treated water, 11.5 μl of 3 M sodium acetate (Ambion; Thermo Fisher Scientific, Dublin, Ireland) and 253 μl ethanol. This mixture was incubated at −20°C for 15 min, before centrifugation at 8,000 rpm (4°C) for 15 min. *In vitro*-transcribed RNA underwent reverse transcription-PCR to make cDNA as outlined above.

To determine target copy number, DNA and RNA were quantified on the NanoDrop 2000C instrument (Thermo Fisher Scientific, Dublin, Ireland). The exact size in base pairs of each gene target was confirmed by nucleotide sequencing of the purified PCR products using the T7 primer. The copy numbers for DNA and RNA standards were calculated assuming a molecular mass of 660 Da for DNA and 330 Da for RNA using the following formula:

$$\frac{\left[6.023 \times 10^{23}\ (\text{copies mol}^{-1}) \times \text{concentration of standard}\ (\text{g } \mu\text{l}^{-1})\right]}{\text{relative molecular mass}\ (\text{g mol}^{-1})} \quad (2)$$

Standard curves were produced by 10-fold dilutions of appropriate DNA or cDNA in RNase-free water. Differences between slope and elevation of the (RT-)Q-PCR standard curves were determined using analysis of covariance (ANCOVA) in Graph Pad Prism v6.

***E. coli* biofilm formation under shear flow.** The BioFlux 1000Z instrument (Fluxion, CA, USA) was used to determine whether pure cultures of the selected *E. coli* strains were capable of (i) attaching to a surface and (ii) forming a biofilm under constant shear stress. Three shear stress conditions, high (4.4 dynes [dyn] cm^{-2}), medium (2.0 dyn cm^{-2}), and low (0.6 dyn cm^{-2}), were tested. The microfluidic channels in the BioFlux 48-well plates (LabTech, Uckfield, United Kingdom) were primed with 50 μl autoclaved LB broth or autoclaved drinking water, as appropriate, from the "outlet" well, and the medium was allowed to flow through the channel for 3 min at a shear force of 5 dyn cm^{-2} until the channels were thoroughly coated, after which excess medium was removed from the wells. Fifty microliters of overnight culture (approximately 10^8 cells ml^{-1}) of each *E. coli* strain (Lys9, J1, or SE11) was added to duplicate outlet wells per experiment, and balanced with 50 μl of medium in the "inlet" well. Overnight stationary-phase cultures were chosen as most species are present in long-term stationary phase in the environment (33). Each strain was pumped from the outlet well into the channel at 1 dyn cm^{-2} until bacteria were visible under the microscope (40×; Zeiss, Oberkochen, Germany). Cell colonization took place at 20°C. After a "settling period" of 1 h, a flow rate of 5 dyn cm^{-2} was applied for 3 min to remove unattached cells. After this, fresh medium, either LB broth or DW, was pumped over the attached *E. coli* at different experimental flow rates: high (4.4 dyn cm^{-2}), medium (2.0 dyn cm^{-2}), and low (0.6 dyn cm^{-2}) for 2.5, 5.5, and 21 h, respectively. The duration of the experiment was determined by the maximum fixed volume of the medium and the flow rate. Biofilm development of *E. coli* was continuously recorded via a high-resolution charge-coupled-device (CCD) camera throughout the experiment. Still images at 0, 2.5, 5.5, and 21 h were captured to compare cell coverage and biofilm development between strains and flow rates at a given time. ImageJ2x was used to divide each image into 66 fields. *E. coli* cells were manually counted within 10 random fields of view at each time point. Cell numbers were averaged and calculated per unit area per time point. Subsequently, the counts from biological replicates ($n = 2$) were averaged. In the control experiment, once aggregates of bacteria made counting single cells difficult, they were reported as "too numerous to count" (TNTC). Changes in cell numbers between time points were reported as a ratio to the starting number.

Growth potential of *E. coli* in drinking water in the presence of pipe wall material. The growth-promoting properties of pipe wall material polyethylene PE-100 on the three *E. coli* strains was assessed using the biomass production potential (BPP) test (34). Representative pieces of PE-100 (six pieces, with a total external surface of approximately 150 cm^2 per test container) were placed in flasks with 600 ml of nonsterilized drinking water and inoculated with a mixture of naturally occurring microorganisms derived from river water and the three strains of *E. coli* (Lys9, J1, and SE11) at a total concentration of 200 CFU ml^{-1}. Noninoculated controls were also included. The flasks were incubated at 30°C for 16 weeks at a constant surface-area-to-volume ratio of 0.16 cm^{-1} and with weekly replacements of the drinking water. Biomass formation of the naturally occurring microorganisms on the pipe wall material and in the water was determined by measuring ATP concentration after 8, 12, and 16 weeks of incubation. The ATP concentration of the water was used to calculate the suspended biomass (ATP pg ml^{-1}). Biomass from the pipe wall material was removed by sonication, from which the ATP concentration was measured and used to calculate the attached biomass (pg ATP cm^{-2}). The biomass production (BP) was determined at 8, 12, and 16 weeks by adding the attached biomass to the suspended biomass multiplied by the volume/surface ratio. The BPP (pg ATP cm^{-2}) was calculated as the average value of the BP values at 8, 12, and 16 weeks minus the BP value for the negative controls on the same days. *E. coli* numbers were monitored by culturing after 14 days from both water and biofilm. *E. coli* was reinoculated (200 to 300 CFU ml^{-1}) on day 27 and tested for culturable *E. coli* 7 days later and on days 56 and 112 from both water and pipe wall biofilm. In addition, on days 0, 14, and 112, 100 μl DNA was extracted from 100 ml of test water; DNA was also extracted from the biofilm which was detached on days 14 and 112 from 25 mm^2 of pipe wall material into 40 ml of water by high-energy sonication. Total 16S rRNA and *E.*

TABLE 2 DNA and RNA standard curve descriptors for all quantitative analysis performed targeting *E. coli* in drinking water[a]

Nucleic acid	Target	Standard curve descriptors					
		Slope	% efficiency	*y*-Intercept	r^2	NTC cycle threshold	NTC copies/μl^{-1}
DNA	16S rRNA (total bacteria)	3.330	99.7	36.90	0.94	29.8	1.13×10^4
	16S rRNA (*E. coli* specific)	5.833	48.4	61.34	0.96	0	0
	uidA	3.458	94.6	42.04	0.97	31.7	1.15×10^3
	tuf	3.472	94.1	34.43	0.97	31.7	3.62×10^3
	rodA	3.528	92.1	43.17	0.98	31.7	3.76×10^3
RNA	16S rRNA (total bacteria)	3.234	103.8	54.40	0.95	26.7	3.92×10^8
	16S rRNA (*E. coli* specific)	6.122	45.7	89.65	0.96	0	0
	uidA	3.268	102.3	58.39	0.91	31.7	8.58×10^7
	tuf	3.602	90	54.44	0.94	31.7	1.58×10^7
	rodA	3.498	93.1	58.47	0.96	31.7	1.05×10^8

[a] To determine the detection limit of each assay, 3.3 cycles were subtracted from the assay's NTC as recommended by Smith and colleagues (20).

coli-specific *rodA* Q-PCR assays were carried out to detect DNA in both the water and material samples as described previously.

Statistical analysis. Differences in cell number, gene/transcript abundance, and T_{90} values between the start and end of individual microcosm treatments were compared using a paired Student *t* test on $\log_{10}(x + 1)$-transformed data (35). Normal distribution of data was confirmed using the Kolmogorov-Smirnov test ($P > 0.05$). Analysis of variance (ANOVA) among strains, temperatures, and water treatments of T_{90} values were investigated using a one- or three-way ANOVA as appropriate, followed by a posthoc Tukey test (36) and Bonferroni's correction (37). A paired Student *t* test was performed on replicate $\log_{10}$ cell counts between the start and end numbers of BioFlux biofilm experiments. All statistical analyses were carried out in IBM SPSS Statistics v21.0.

RESULTS

Test panel specificity and (RT-)Q-PCR assays. In order to select a Q-PCR assay and subsequently design RT-Q-PCR assays for *E. coli* with the highest levels of specificity, sensitivity, and efficiency, a literature review identified three frequently used functional genes for *E. coli*, *uidA* (24), *tuf* (25), and *rodA* (26) for comparison. The *uidA* gene (coding for β-D-glucuronidase) was chosen as the most frequently used target gene for *E. coli* (24, 38, 39, 40, 41). Its specificity, however, has been questioned (27), and therefore, two other promising gene targets, *tuf* (coding for a protein elongation factor) (25), present in one to two copies, and *rodA* (coding for a unspecified protein, affecting cell shape and amdinocillin) (26), were also selected. *rodA* was included as a single-copy gene target and therefore had the potential to act as a molecular proxy for an *E. coli* cell count. The 16S rRNA gene primer set was selected as a primer set that discriminated between *E. coli* and *Shigella* spp., with the potential to act as a quantitative marker for active *E. coli* by applying it as a RT-Q-PCR on 16S rRNA gene transcripts, as the 16S rRNA gene is constitutively expressed in active cells.

The specificities of the Q-PCR assays (Table 1) were confirmed using a panel of test strains (see Table S1 in the supplemental material). The *uidA*, *tuf*, and *rodA* gene primer and probe sets amplified only *E. coli* and the three tested *Shigella* species. The *E. coli*-specific 16S rRNA gene primers (27) differentiated between *E. coli* and the three *Shigella* species, but this was achieved only by using a high annealing temperature (72°C) as previously reported (27). A SYBR green Q-PCR assay was developed for the 16S rRNA gene primer set. Furthermore, RT-Q-PCR standard curves were constructed, and assays were optimized for each of the gene targets. Standard curve descriptors for both DNA and RNA assays used in this study are listed in Table 2. All efficiencies were between 90 and 105%, commonly regarded as acceptable standard curve efficiency (the ideal being 100%), except for the 16S rRNA *E. coli*-specific assays at both the DNA and cDNA level. In order to retain the specificity of the reaction, a high annealing temperature (72°C) was required, which in turn, rendered the efficiency of the standard curve below par (Table 2). Any improvements to the efficiency of the assay were at the cost of a loss of specificity. Therefore, it was decided to keep the annealing temperature at 72°C. Excluding the 16S rRNA (*E. coli*-specific) assays, the *y*-intercept values fall between 34.43 and 43.17 for DNA assays, and between 54.40 and 58.47 for RNA assays. Standard curves for each gene were significantly different from each other ($P < 0.05$ by *t* test). The variations observed in *y*-intercept values of each assay (Table 2) indicate variation in the absolute numbers obtained from each individual standard curve (20, 21).

***E. coli* survival in drinking water microcosms. (i) Culture-based quantification of *E. coli* persistence.** Viable *E. coli* cells were recovered from all three strains 70 days after the initial inoculation (see Fig. S1 in the supplemental material). Significantly lower cell numbers were recorded between the start and end at 17°C ($P < 0.05$ by *t* test), with the exception of *E. coli* Lys9 in FA-DW, and for all strains and treatments at 8°C ($P < 0.05$ by *t* test), except for *E. coli* J1 in FA-DW ($P > 0.05$ by *t* test). However, T_{90} varied greatly among strains and treatments (Table 3). A

TABLE 3 T_{90} inactivation time for each *E. coli* strain in DW and FA-DW at 8 and 17°C

E. coli strain	Water type	T_{90}[a] (days) at:	
		8°C	17°C
Lys9	DW	**39.9 ± 14.3**	18.2 ± 1.0
	FA-DW	**99.7 ± 18.7**	93.5 ± 29.9
J1	DW	17.4 ± 1.8	**16.3 ± 2.0**
	FA-DW	31.5 ± 7.0	**28.3 ± 4.2**
SE11	DW	24.4 ± 7.5	**149 ± 67.7**
	FA-DW	71.9 ± 10.3	**97.3 ± 34.0**

[a] Values are means ± standard deviations (SD) ($n = 3$). Statistical differences between each individual strain (Lys9, J1, or SE11) and water type (FA-DW or DW) are indicated in boldface font, whereas statistical differences between strains and temperature (8 or 17°C) are underlined ($P < 0.05$ by *t* test).

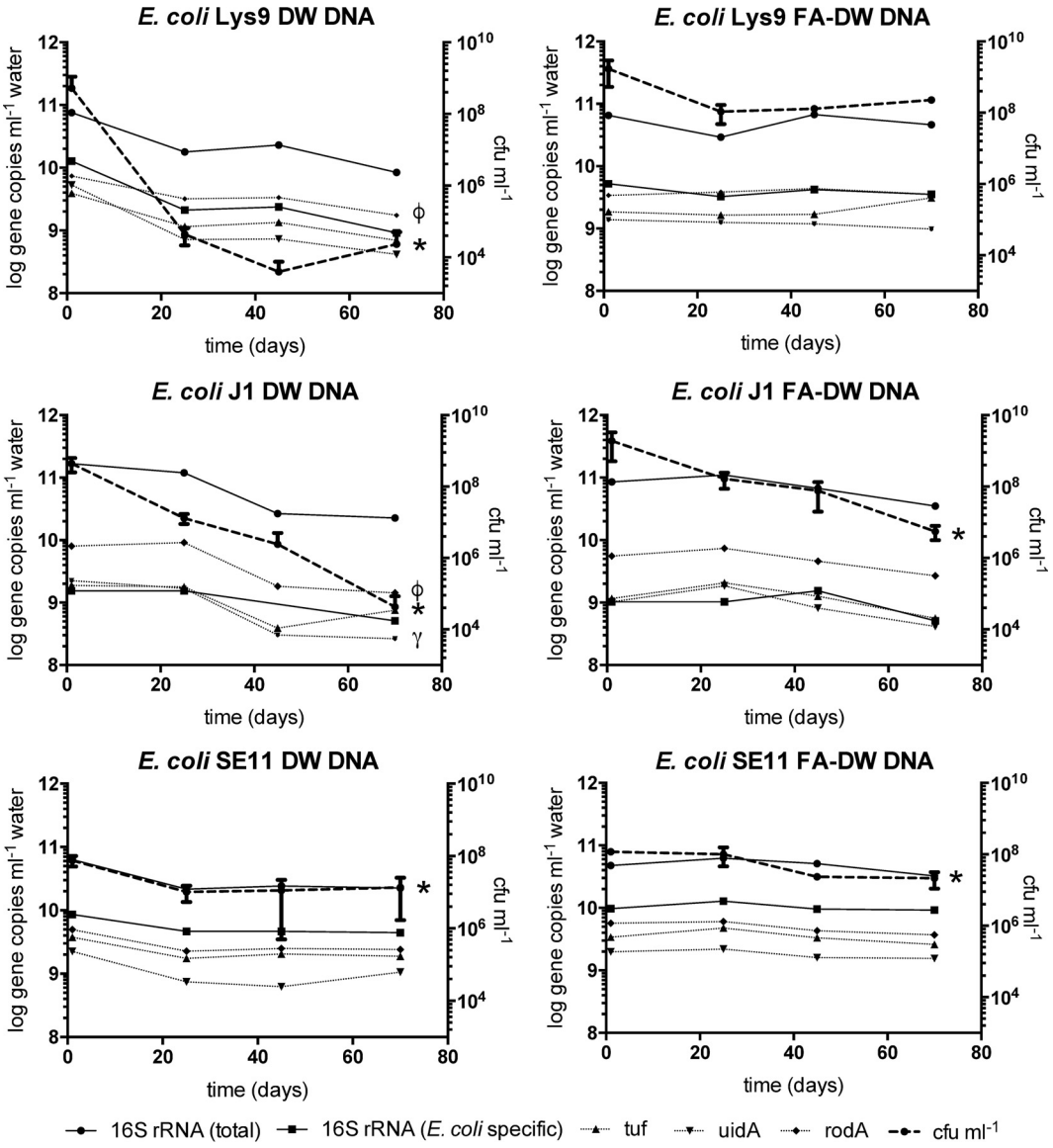

FIG 1 *E. coli* (Lys9, J1, and SE11) cell and gene abundances ml^{-1} water in DW and FA-DW at 17°C over 70-day incubation. The number of gene copies $milliliter^{-1}$ water of 16S rRNA (total), 16S rRNA (*E. coli* specific), *uidA*, *tuf*, and *rodA* are plotted on the left *y* axis. The number of CFU $milliliter^{-1}$ are plotted on the right *y* axis. The error bars represent standard deviations ($n = 3$). Significant differences between start and end CFU ml^{-1} are indicated by an asterisk (*), whereas significant differences between start and end gene abundances are marked with either γ (*uidA*) or ϕ (*rodA*) ($P < 0.05$ by *t* test). No significant differences were noted between start and end *tuf* gene abundances for any treatment ($P > 0.05$ by *t* test).

three-way ANOVA comparing T_{90} values between strains, treatments, and temperatures showed significant difference among *E. coli* strains and water treatments ($P < 0.05$), but not temperature ($P > 0.05$). T_{90} values were consistently higher in the FA-DW microcosms than in the corresponding DW microcosms (1.8 to 5.1 times longer), with the exception of strain SE11 at 17°C. This difference between water types was statistically significant only for the Lys9 microcosm at 8°C and the J1 and SE11 microcosms at 17°C ($P < 0.05$ by *t* test). For individual strains, temperature had no effect on the persistence of strain J1 or Lys9 ($P > 0.05$ by *t* test). In contrast, the T_{90} of strain SE11 was significantly longer at 17°C in the DW microcosm ($P < 0.05$ by *t* test). There was no difference between the persistence of the three *E. coli* strains at 8°C in DW ($P > 0.05$ by one-way ANOVA), whereas in the FA-DW, the J1 T_{90} values were significantly different from those of Lys9 ($P < 0.05$) and SE11 ($P < 0.05$). At 17°C, however, the SE11 T_{90} values were statistically significant from the two other strains in DW ($P < 0.05$ by one-way ANOVA), while in the FA-DW, Lys9 was similar to both J1 and SE11 ($P > 0.05$), but J1 and SE11 T_{90} values were significantly different from each other ($P < 0.05$ by one-way ANOVA).

(ii) Molecular quantification of *E. coli* persistence. Four *E. coli*-specific and one universal bacterial Q-PCR and RT-Q-PCR assays (Table 1) were used to quantify *E. coli* gene and transcript abundances in the 17°C microcosms. While culturable cell numbers reduced with time to different extents between treatments (Table 3), gene abundances, determined by the five Q-PCR assays (Fig. 1), were statistically similar over the 70-day period ($P > 0.05$

by one-way ANOVA). However, *E. coli* Lys9 DW *rodA* and *E. coli* J1 DW *rodA* and *uidA* gene abundances were significantly lower than their starting number after 70 days ($P < 0.05$ by *t* test). Absolute gene abundances varied between Q-PCR assays due to variation in the standard curves (Table 2).

RNA transcripts of each assay were targeted by RT-Q-PCR to determine whether this was a suitable approach to detect viable *E. coli*. The numbers of 16S rRNA transcripts per ml of water quantified via the *E. coli*-specific assay were greater than the numbers of 16S rRNA transcripts per ml of water quantified via the universal bacterial assay (Fig. 2); this can be attributed to differences in the efficiencies of the RT-Q-PCR assays (Table 2). However, the patterns were the same. 16S rRNA transcripts were quantified throughout the experiment for all strains at each temperature, and, unlike the cell counts, they did not decrease over time ($P > 0.05$ by *t* test). While 16S rRNA gene transcripts were quantified throughout the experiment, *tuf*, *uidA*, and *rodA* gene expression was not consistently quantified throughout the 70-day microcosm experiment (Fig. 2). The functional gene targets did not make reliable markers for viable cells, as they were present throughout the experiment, as determined by the cell count.

***E. coli* biofilm formation under shear stress.** The ability of each strain to attach to a surface and form a biofilm under flow rates similar to those experienced in a WDS (0.6, 2.0, and 4.4 dyn cm^{-2}, equivalent to 0.06, 0.2, and 0.44 N m^{-2}, respectively) was examined using the BioFlux (Fluxion) system. After an initial settling period (1 h at 20°C) in the BioFlux chamber, a flow rate of 5 dyn cm^2 was applied for 3 min to remove unattached cells. All cells that remained were regarded as attached to the surface. Each of the three *E. coli* strains attached to the glass slide surface of the BioFlux chamber (0 h in Fig. 3 and in Fig. S2 in the supplemental material). Subsequently, nutrient-rich LB broth (control) (optimum, nutrient-rich environment for cell growth) or DW flowed over the attached *E. coli* cells at the low, medium, and high steady-state shear stresses (0.6, 2.0, and 4.4 dyn cm^{-2}) to determine whether the attached cells could multiply, while attached, forming a biofilm. When LB broth was used as the growth medium, an increase in cell coverage on the slide was seen ($P < 0.05$ by *t* test), indicating that all three strains were capable of multiplying while attached to a surface, forming biofilm at a range of shear stresses in the presence of nutrients (Fig. S2). However, when drinking water flowed over the attached cells, there was no statistical increase ($P > 0.05$ by *t* test) in cell number per mm^2 (Fig. 3). At the lower flow rates, cells remained attached, but when the flow rate was increased to the highest shear (4.4 dyn cm^{-2}), attached cells were removed from the surface and resuspended in the bulk liquid. At the end of each experiment, it was tested whether the attached cells could be removed by a further increase in shear stress as would occur with a leak or breakage in a WDS. All traces of *E. coli* cells were removed from the glass surface when the shear stress was increased to 16 dyn cm^{-2} for 40 min (data not shown).

Growth of *E. coli* in the presence of WDS pipe wall material is not enhanced. The growth-promoting properties of a commonly used pipe wall material (PE-100) in WDS was tested to determine whether it enhanced the growth of *E. coli* in either biofilm or drinking water. BPP was measured as a proxy for total microbial activity in drinking water containing PE-100 pipe wall material (34). The BPP values were comparable between the PE material inoculated with a river water inoculum (592.5 ± 230.9 pg ATP cm^{-2}) and the PE material inoculated with the three *E. coli* strains and a river water inoculum (690.4 ± 107.6 pg ATP cm^{-2}), indicating that growth was not enhanced by adding the three *E. coli* strains. Water and biofilm material were plated for *E. coli* on days 0, 7, and 14, and *E. coli* was recovered only on day 0 (immediately after inoculum addition). Subsequently, *E. coli* cells were reinoculated on day 29 in the BPP test, and cultivable *E. coli* cells were detected immediately after reinoculation (day 29), but not on days 52 and 112. Q-PCR data using the *rodA* gene assay supported this observation (Table 4), indicating that growth of *E. coli* did not occur and survival times in natural drinking water with biofilms are low.

DISCUSSION

This study set out to determine whether *E. coli*, represented by two enteric isolates and one soil isolate, could survive in drinking water and form biofilms under shear stress, and, most importantly, to evaluate their potential for regrowth in drinking water in a series of laboratory-controlled experiments.

Survival. The *E. coli* strains selected persisted beyond the 70-day experiment, with greater persistence evident in the sterile microcosms in most cases and with T_{90} values indicating survival for considerably long periods in either DW or FA-DW. This is significant for public health microbiology, as in all cases, it was beyond the typical short retention times of a WDS (42). Retention times are strongly associated with the characteristics and operation of an individual WDS (43), typically varying between 24 h, which is the average hydraulic retention time in the United Kingdom (44) and 48 h (45), with longer retention times increasing the number of bacteria in the bulk water (46). Survival results are in line with those reported in similar freshwater aquatic environments ranging from 12 to 260 days (47, 48, 49, 50). In general, extended survival was enhanced by sterilization of the water prior to inoculation (47, 48, 51, 52), indicating the role of grazing protozoa (47) and/or competition for limited resources with the indigenous microbial community (53). Survival was also strain dependent and influenced by temperature in the nontreated drinking water. For example, the T_{90} value at 8°C was greater for the soil isolate Lys9 than for the corresponding enteric strains. Previous survival experiments in water have focused primarily on *E. coli* O157:H7 and have not considered environmental isolates. However, similar observations were made for a range of *E. coli* isolates from swine manure slurry that exhibited different survival times in manured soils (54). While it is commonly accepted that higher temperatures enhance *E. coli* growth (55, 56), longer persistence of *E. coli* at lower temperatures (5 and 8°C) has been shown (57, 58). This may favor *E. coli* survival, as it slows the metabolism of the *E. coli* bacteria, lowers their affinity for substrates (59), and/or may reduce competition with the natural microbial community. Yet, the effect of temperature on survival was strain dependent; therefore, strain type seems to be a greater determinant of persistence in the environment than temperature. It has been previously suggested that differences within the *E. coli* genome might correlate with survival/persistence in the environment (53).

Molecular detection. Discrepancies between the total heterotrophic cell counts and gene abundances could be due to a number of reasons such as the *E. coli* cells entering a VBNC state upon inoculation into drinking water (60). No differentiation can be made between DNA from viable and nonviable cells. While all of the functional gene assays used were suitable for quantification,

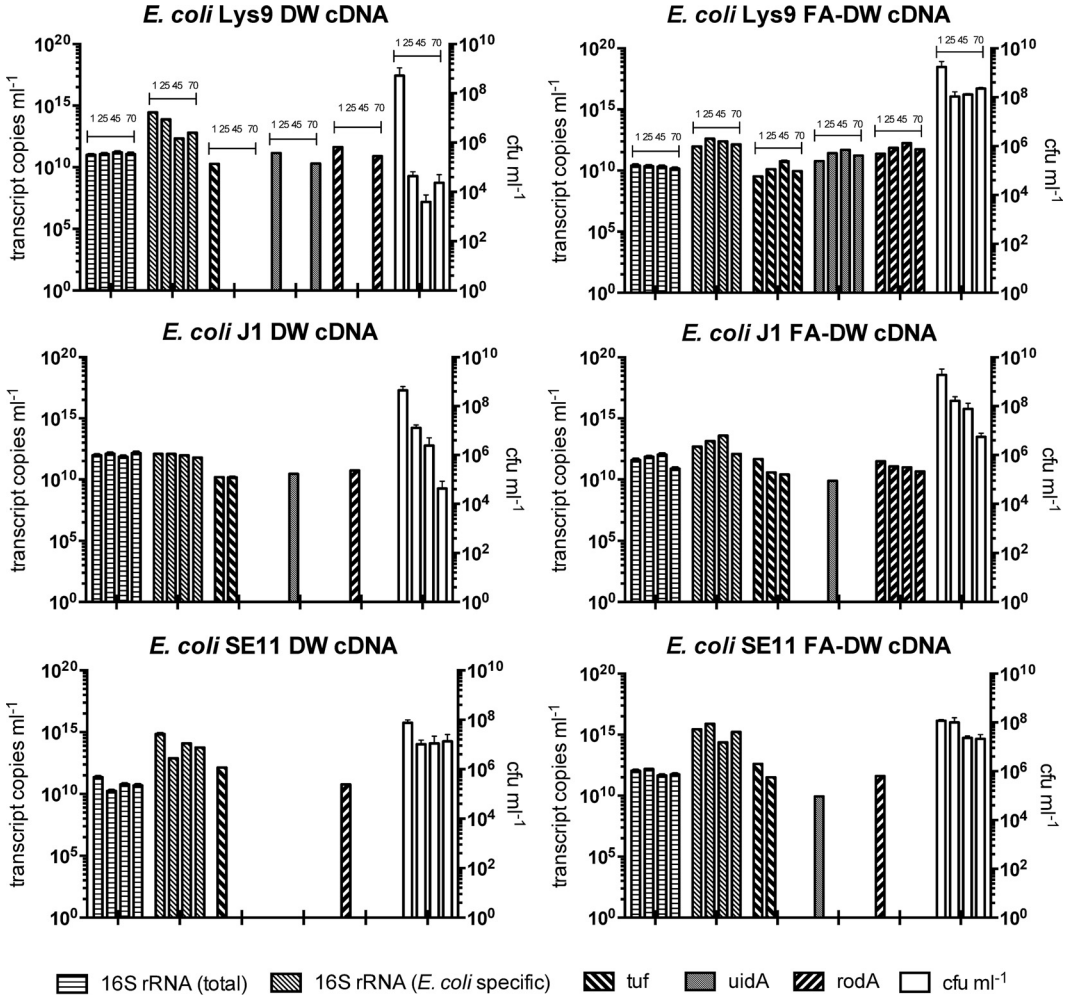

FIG 2 Transcript copy numbers milliliter^{-1} of water for *E. coli* Lys9, J1, and SE11 in both DW and FA-DW quantified from days 1, 25, 45, and 70. The number of transcript copies milliliter^{-1} water of 16S rRNA (total), 16S rRNA (*E. coli* specific), *uidA*, *tuf*, and *rodA* are plotted on the left *y* axis. The number of CFU milliliter^{-1} are plotted on the right *y* axis. No RNA was detected by RT-Q-PCR where bars are not present. The error bars represent standard deviations between triplicate samples. In all cases, start and end 16S rRNA transcript abundances were statistically similar ($P > 0.05$).

intrinsic problems have been associated with the *uidA* gene as a specific target for *E. coli* detection (27), and the *tuf* gene may have one or two copies in *E. coli* (61); as such, we selected and recommend the *rodA* gene Q-PCR assay for subsequent use.

RT-Q-PCR assays were developed and tested as markers for viable cells. None of the functional gene markers were suitable for detecting viable cells, as selected marker genes were not constitutively expressed throughout the duration of the microcosm experiment. However, despite the low efficiency of the *E. coli*-specific 16S rRNA assay, we propose that it has potential to be used as a sensitive presence/absence indicator of viable *E. coli* cells in drinking water. The detection of 16S rRNA transcripts is a stronger indication of viable *E. coli* than DNA alone, as the presence of *E. coli* 16S rRNA transcripts in drinking water indicates the potential for protein synthesis within these cells (62). In this case, the absolute number of transcripts may be less important than their detection, which indicates the presence of *E. coli* bacteria that are either currently active or have been active in the recent past (63).

Biofilm. A further concern for the retention of potential fecal indicators and/or pathogens within WDS is their ability to incorporate into biofilm. This is an issue for the microbiological quality of drinking water, working on the widespread assumption that biofilms are a major source of microbes even in WDS that have undergone satisfactory water treatment and do not suffer from leaks or breakages (64). We have demonstrated that monocultures of *E. coli* can attach to a surface and multiply to form a biofilm under shear stress in the presence of nutrient-rich LB broth (control), but not drinking water. Throughout the experiment, the cells remained attached, except at the highest shear stress (4.4 dyn cm^{-2}) where cell numbers decreased as they were removed and reinoculated into the bulk liquid. Previously, two laboratory-scale studies focused on drinking water biofilms reported *E. coli* incorporation into the matrix (65, 66) and concluded that *E. coli* did grow within the biofilm. Fass and colleagues concluded that *E. coli* incorporated into the matrix, as *E. coli* cell numbers in the biofilm increased above the predicted values (65), whereas Williams and Braun-Howland detected small, metabolically active *E. coli* cells within a forming biofilm (66). Furthermore, two culture-independent studies of biofilm retrieved from WDS using different variations of fluorescent *in situ* hybridization (FISH) quantified *E.*

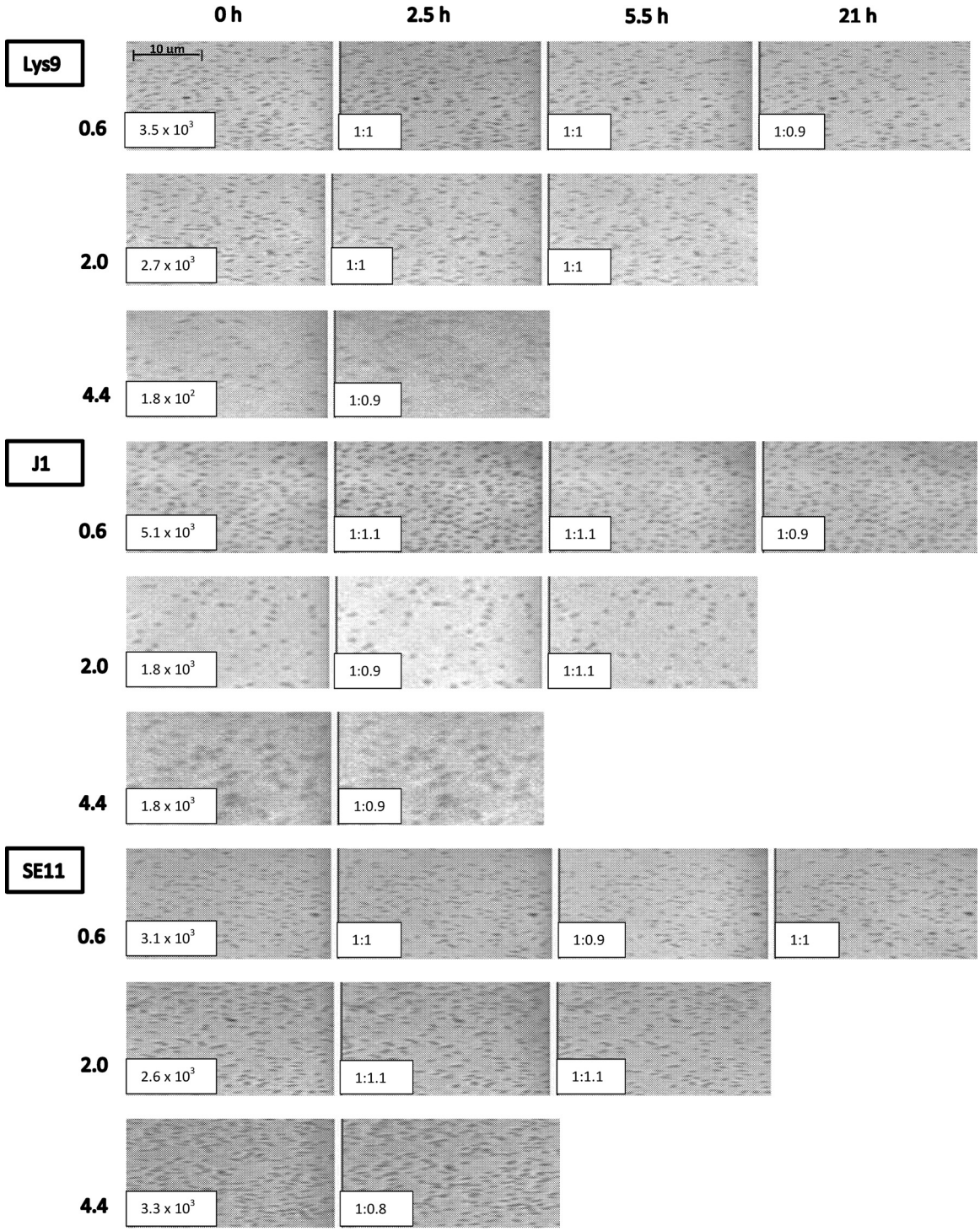

FIG 3 Attachment and biofilm accumulation under shear flow conditions. *E. coli* Lys9, J1, and SE11 with DW flow rates of 0.6, 2.0, and 4.4 dyn cm^{-2}. The times 0, 2.5, 5.5, and 21 h are representative of the length of each shear stress experiment on the BioFlux system. Bright-field images were captured at a ×40 magnification at the time points indicated and are representative of two independent experiments. At 0 h, the number of *E. coli* cells per mm^2 is given; subsequently, each time point thereafter is compared by way of a ratio to the initial starting number. Student's *t* test was used to identify statistical differences in the number of attached cell numbers between the start and end of the experiment ($P > 0.05$).

coli within biofilms and estimated that they contributed between 0.001 and 0.1% of total biofilm microbial community (8, 67), but Juhna and coworkers noted that it was unlikely that the cells were growing due to the sparse distribution of individual *E. coli* cells within the biofilm (8). The detection of *E. coli* within the pipe wall biofilm raises two possibilities—are the biofilms simply accumulating *E. coli* or is there regrowth?

Regrowth. To determine whether the chosen *E. coli* strains

could grow in drinking water and/or drinking water biofilms in the presence of an indigenous microbiota, *E. coli* strains were inoculated into microcosms in the presence of pipe wall material. Pipe wall material was included, as carbon compounds leaking from the plastic can enhance the growth of microorganisms and pathogens (68, 69). PE-100 was selected as an intermediate material in terms of promoting bacterial growth (34) and because it is regularly used in WDS. *E. coli* cells were inoculated at lower cell densities than in the survival experiment where cell densities were high (10^8 CFU ml^{-1}) (47) to ensure that die-off could be monitored over the 70-day experimental period. In order to test the regrowth potential of *E. coli*, it was essential that cell densities were lower and in line with the carrying capacity of the water (56) defined as "the maximum population of a species that a habitat can support without permanently impairing the habitat's productivity" (70). The results of the *E. coli* plate counts and Q-PCR assays clearly showed that the *E. coli* strains did not grow in biofilm or bulk drinking water, nor did they colonize the pipe wall biofilm in the presence of an indigenous microbial community. While we have not demonstrated whether *E. coli* regrowth could be supported by carbon compounds from PE-100 under sterile conditions, this was not our aim, as it is not a scenario that would be encountered in a WDS. It was our objective to determine whether *E. coli* regrowth occurred in the presence of an indigenous microbial community, since *E. coli* encounters these conditions when it has entered the WDS. Further studies are thus required to determine whether *E. coli* can use the carbon compounds from pipe wall material to support its growth. *E. coli* O157 has been shown to grow in sterile drinking water at a lower specific growth rate than the natural microbial community, but not when a natural drinking water microbial community is present (71). In this study and in WDS, it is likely that the natural microbial community present outcompetes *E. coli* for the available AOC (56, 71) and probably also in the biofilm. This is likely due to less well-adapted kinetic growth properties of *E. coli* versus the natural drinking water microbial community, which is adapted to growth at low organic carbon concentrations (56).

In the regrowth experiments reported in this study, the *E. coli* strains did not grow in the bulk water or in biofilm on PE-100 in the presence of an indigenous microbial community. While we have shown that *E. coli* can persist for extended periods in drinking water when high numbers are inoculated, such as in a fecal pollution event, our results indicate that the potential for *E. coli* regrowth in drinking water is low. *E. coli* is not adapted in terms of specific growth rates to the oligotrophic drinking water environment, and when it is challenged by natural water microbes with higher specific growth rates, it will be outcompeted for the limiting resources. The results from the BPP test also demonstrate that within these drinking water microcosms, in the presence of an indigenous microbial community and PE-100 pipe wall material, and at a surface-to-volume ratio of 0.16 under static conditions, cultivable *E. coli* and *E. coli* DNA disappear between 0 and 14 days. Additional research is required to determine the exact removal time for *E. coli* under these conditions.

Conclusion. Overall, this study has shown the following. (i) The *E. coli* strains tested can persist in drinking water for extended periods. (ii) While *E. coli* cells did attach to a surface and form a biofilm under shear stress in nutrient-rich conditions in the absence of a natural microbial community, cells did not replicate when only drinking water was supplied, but some were resus-

TABLE 4 Q-PCR data from 16-week incubation of combined *E. coli* incubated in the presence of pipe wall material

Pipe wall material or culture	No. of gene copies ml^{-1} (mean ± SD) ($n = 2$)[a]									
	Day 0, water		Day 14				Day 112			
			Water		Material		Water		Material	
	Total 16S rRNA	*rodA*	Total 16S rRNA	*rodA*	Total 16S rRNA	*rodA*	Total 16S rRNA	*rodA*	Total 16S rRNA	*rodA*
Glass	$8.06 \times 10^8 \pm 1.44 \times 10^8$	ND	$4.86 \times 10^8 \pm 5.11 \times 10^8$	ND	$1.60 \times 10^6 \pm 3.54 \times 10^4$	ND	$4.04 \times 10^8 \pm 1.06 \times 10^7$	ND	$7.50 \times 10^5 \pm 1.06 \times 10^6$	ND
PVC	$7.08 \times 10^8 \pm 2.33 \times 10^7$	ND	$1.21 \times 10^{11} \pm 3.54 \times 10^{10}$	ND	$2.12 \times 10^9 \pm 6.79 \times 10^8$	ND	$1.06 \times 10^{10} \pm 4.95 \times 10^8$	ND	$3.95 \times 10^8 \pm 1.82 \times 10^8$	ND
E. coli + inoculum	$7.99 \times 10^8 \pm 7.2 \times 10^7$	$6.12 \times 10^5 \pm 1.07 \times 10^4$	$3.56 \times 10^9 \pm 2.15 \times 10^9$	ND	$8.64 \times 10^6 \pm 1.41 \times 10^6$	ND	$6.39 \times 10^9 \pm 1.05 \times 10^9$	ND	$8.48 \times 10^6 \pm 3.56 \times 10^6$	ND
E. coli − inoculum	$5.66 \times 10^8 \pm 6.22 \times 10^7$	$6.03 \times 10^5 \pm 7.71 \times 10^4$	NM	NM	NM	NM	$6.73 \times 10^8 \pm 1.29 \times 10^8$	$2.03 \times 10^6 \pm 3.01 \times 10^6$	$6.06 \times 10^6 \pm 4.05 \times 10^6$	ND

[a] ND, not detected; NM, not measured.

pended in the water under high shear stress. (iii) Regrowth of *E. coli* in drinking water in the presence of PE-100 pipe wall material with a natural microbial community present was not supported. This indicates that while *E. coli* can persist within a WDS, regrowth of *E. coli* in WDS is unlikely to be supported. Furthermore, expanding on current DNA-based approaches, an *E. coli*-specific 16S rRNA gene RT-Q-PCR assay is presented for use as a sensitive and specific presence/absence marker for viable or recently active *E. coli* within WDS.

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